AD_____

Award Number: DAMD17-99-1-9151

TITLE: Murine Models of Breast Cancer: Assessment of the Role of c-Src in Mammary Tumorigenesis

PRINCIPAL INVESTIGATOR: Konstantina Alexandropoulos
Luzhou Xing
Rebecca Huber

CONTRACTING ORGANIZATION: Columbia University
New York, New York 10032

REPORT DATE: October 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2001	Annual (01 Oct 00 - 30 Sep 01)

4. TITLE AND SUBTITLE
Murine Models of Breast Cancer: Assessment of the Role of c-Src in Mammary Tumorigenesis

5. FUNDING NUMBERS
DAMD17-99-1-9151

6. AUTHOR(S)
Konstantina Alexandropoulos
Luzhou Xing
Rebecca Huber

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Columbia University
New York, New York 10032

E-Mail: ka141@columbia.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Strong evidence exists that the c-Src non-receptor tyrosine kinase plays a role in the pathology of human breast tumors. The purpose of this study is to examine the role of c-Src in mammary tumorigenesis and elucidate the mechanisms that lead to tumor formation in an animal model for breast cancer. In our previous experiments we used Src substrates that we cloned to activate c-Src and study its signaling mechanisms. Using one of these substrates, Sin, we characterized a signaling cascade that is activated as a result of Sin binding to Src and Src-mediated Sin phosphorylation. We found that Src-phosphorylated Sin recruits a signaling complex that leads to the activation of the small GTP-binding protein Rap1. Rap1 then activates the ERK kinase that in turn mediates Src-dependent transcriptional activation. In addition, when we compared Sin activated wild-type Src and oncogenic Src protein we found that their signaling mechanisms differed in that, wild type Src signaling is mediated by the Rap1 cascade whereas oncogenic Src signaling is mediated by Rap1, as well as another G-protein, Ras. Our results for the first time implicate the Rap1 pathway in wild type and oncogenic Src signaling and reveal mechanistic differences in the signaling mechanisms of these proteins. In our future experiments we will address the role of Rap1 activation in cellular transformation and mammary tumorigenesis in cell lines and transgenic mice coexpressing Src and Sin. These experiments will provide insight into the mechanisms of Src-mediated tumorigenesis and may lead to the identification of proteins that will be used as targets for drug development.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast cancer	11
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover.....1

SF 298..... 2

Table of Contents.....3

Introduction.....4

Body.....4

Key Research Accomplishments.....8

Reportable Outcomes.....9

Conclusions.....10

References.....10

Appendices.....11

INTRODUCTION

We are studying the role of the non-receptor tyrosine kinase c-Src in mammary tumorigenesis in a murine animal model and in cell lines. Our purpose is to use c-Src substrates that we have isolated to gain insight into the mechanisms that regulate the activity of c-Src and define the molecular interactions that mediate Src-dependent intracellular signaling and transformation. The molecules we developed bind to a conserved regulatory region of c-Src activate Src's enzymatic activity and subsequently act as substrates and effector molecules for c-Src signaling. In our experiments we are focusing on one novel protein that we isolated, Sin, to identify factors which play a role in Src-mediated tumorigenesis in the mammary epithelium. We are studying Sin because in our experiments we found that this protein has the unique ability among the other c-Src ligands to activate the transforming potential of c-Src. In addition, a modified version of full length Sin was recently identified as a cDNA clone whose expression is upregulated in mouse mammary tumors. Thus, in our studies we will a) express c-Src and Sin variants in the mammary epithelium and mammary epithelium-derived cells lines to assess the effect of these proteins on mammary tumorigenesis. b) Identify Sin sequences that are important for Sin function and use these sequences as a means to isolate proteins that mediate Sin-dependent c-Src tumorigenesis. Given that c-Src has been implicated in the development of human breast tumors, these studies are important because they will elucidate the molecular mechanisms that drive mammary tumorigenesis and may lead to the development of strategies to interfere with aberrant Src activity and Src-induced carcinogenesis. In addition, these studies may implicate endogenous Sin as a novel regulator of mammary tumorigenesis.

BODY

c-Src is a non-receptor tyrosine kinase that is very important for cellular function (2). The Src substrate Sin that we have characterized is a multi-adapter molecule that mediates the formation of multi-protein complexes in a phosphotyrosine-dependent manner (1). Because phosphorylated Sin has the ability to bind to different proteins simultaneously, it also has the potential for activating multiple intracellular pathways with pleiotropic effects on cellular behavior. In our preliminary results we found that a truncated version of Sin, SinΔC, activates two major signaling pathways and the transforming potential of c-Src.

Given the effects of SinΔC on Src activation, in aim 1 of the application we proposed to generate transgenic mice coexpressing Src and SinΔC in their mammary epithelium and examine whether expression of these proteins would result in tumors.

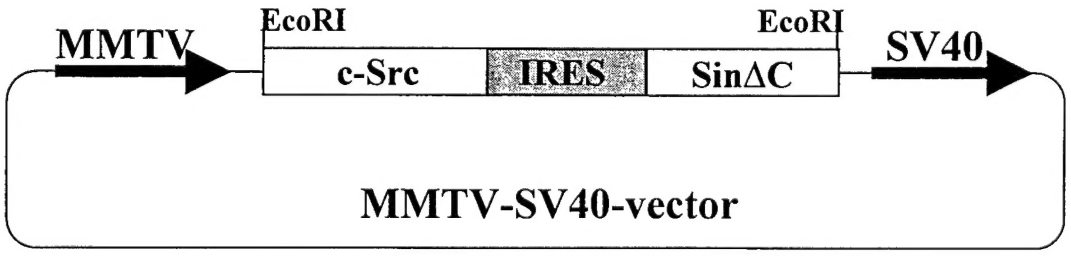

Fig. 1. *Transgenic construct coexpressing c-Src and SinΔC.* This construct is generated by inserting an EcoRI DNA linear fragment containing Src- and SinΔC-expressing cDNAs as well as an internal ribosome entry site (IRES) that allows coexpression of Src and SinΔC, into the EcoRI linearized MMTV vector. MMTV is the long terminal repeat of the mouse mammary tumor virus that allows expression of cloned cDNAs and SV40 is the polyA/splice of SV40.

We have now generated this construct and a linearized fragment of the MMTV vector containing the sequences MMTV-c-Src-IRES-SinΔC-SV40 was recently microinjected into fertilized eggs from hyperovulated donor females. The generation of the trangenic lines took longer than initially anticipated due to cloning problems and delays in microinjecting due to Mouse Hepatitis Virus (MHV) outbreak in the animal facilities of Columbia University. The progeny of the injected animals is currently being analyzed for incorporation and expression of the Src/SinΔC transgenes by PCR, Southern and Western blots. Transgenic progeny that coexpress Src and SinΔC will be bred to themselves (for increased expression of the Src/SinΔC transgenes) or to wild type animals and the F1 progeny will be followed and observed for tumor formation as described in the original application.

Recently, we identified a variant form of Sin by screening an expressed sequence tag (EST) database. This database is compiled by the National Cancer Institute and is part of the Cancer Genome Anatomy Project (CGAP) which was created to determine the gene expression profiles of normal, pre-cancer, and cancer cells. The Sin variant was cloned from a mouse mammary tumor and its expression was found to be upregulated in the tumor as compared to normal mammary epithelium. Most of the Sin variant is similar to the full length Sin protein that we previously described (Alexandropoulos and Baltimore). Full length Sin consists of an N-terminal SH3 domain, a central region with multiple tyrosine containing motifs, two proline-rich regions that bind to the Src SH3 domain and a conserved C-terminus with unknown function (Fig. 2). On the other hand, although the mammary tumor variant Sin protein (Sin-MTV) is mostly similar to full length Sin, it contains a deletion at the C-terminus that removes half of the conserved 150 C-terminal amino acids of full length Sin. In addition, the deleted sequences are replaced with fifty novel amino acids derived from the 3' untranslated region of Sin by alternate splicing (Fig. 2).

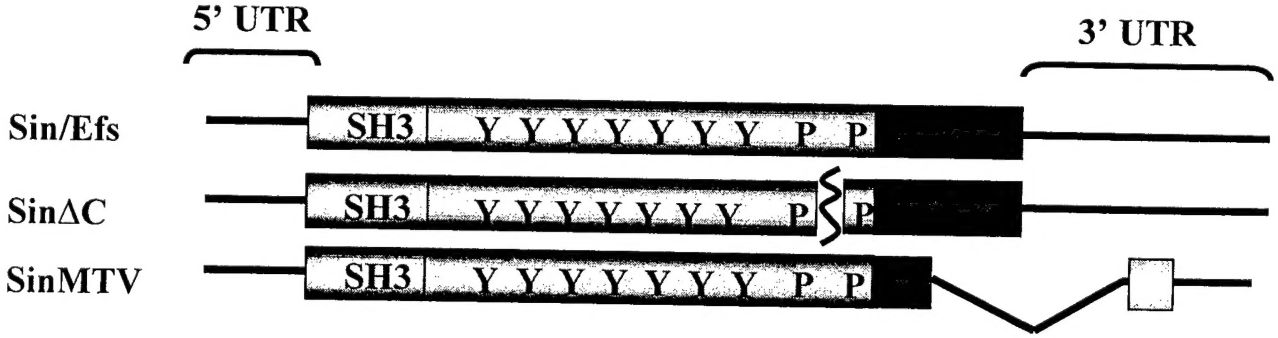

Fig. 2. *Schematic representation of wild type and modified Sin proteins.* SH3 (Src homology region 3) represent the conserved, proline-binding domain of Sin. Y represents tyrosine containing motifs found in the central, substrate-binding region of Sin. P represents the proline-rich motifs of Sin that interact with theSrc SH3 domain. UTR stands for untranslated region.

In previous and ongoing studies we have shown that the conserved C-terminus of Sin has an inhibitory effect on the ability of full length Sin to activated Src kinase-mediated signaling since deletion of the C-terminus, in the case of SinΔC, potentates Src activation and signaling. This observation, together with the existence of the Sin-MTV protein, suggests an important role for the C-terminus of Sin in Sin function. Thus, in addition to SinΔC, it will also be important to address the role of the Sin-MTV protein in mammary tumorigenesis given that this protein is overexpressed in mammary tumors. To this end, we have obtained this the Sin-MTV cDNA clone and we are in the process of characterizing its function.

We will first examine the tumorigenic potential of the Sin-MTV protein in mammary epithelium-derived cell lines. The Sin-MTV cDNA will be cloned into a vector under the control of the murine mammary tumor virus (MMTV) promoter that allows expression of cloned cDNAs in the mammary epithelium and mammary epithelium-derived cell lines. The protein will be expressed either alone or with c-Src and will be tested for its ability to promote transformation of immortalized mammary epithelium-derived cell lines (184B5 and/orMCF10A). Similar experiments will also be performed with SinΔC and Src in the 184B5/MCF10A cell lines, since this is a more relevant system than the NIH3T3 fibroblasts used in our previous experiments. These experiments will be performed in parallel with the characterization of the Src/SinΔC transgenic lines mentioned above. In addition we will generate transgenic mice coexpressing Src and Sin-MTV as mentioned above for Src and SinΔC.

In aim 2 of the original application we proposed to analyze the sequences of Sin to identify elements that are important for the signaling properties of the protein and use these sequences as probes to identify proteins that mediate Sin-dependent Src signaling. In a manuscript we published last year (included in the appendix) we have characterized the signaling mechanisms of Sin and have identified proteins that are involved in mediating Src signaling using the human embryonic carcinoma 293HEK cell culture system (3). In this study, through the use of mutagenesis analysis and dominant negative inhibitors, we found that Src phosphorylated Sin forms a signaling complex that consists of the adapter protein Crk and C3G, a nucleotide exchange factor that promotes guanyl-triphosphate (GTP) binding on the GTP-binding protein Rap1. Binding of GTP to Rap1 activates this protein. Rap1 then activates another kinase ERK1 that in turn is required for transcriptional activation. Activation of both Rap1 and ERK1 is required for Sin-mediated Src signaling. Our results for the first time implicate this pathway in c-Src signaling and provide insight into the signaling mechanisms of wild type Src.

In parallel experiments we aimed to address the in vivo role of Sin. To this end we are using organs of the immune system (thymus and spleen) as model tissues to address the biological function of Sin. These experiments are relevant since Sin is highly expressed in the thymus and to a lesser extent in the spleen. Using transgenic animals that express SinΔC in their thymi and T cells we found that expression of SinΔC in thymocytes and T cells has a negative effect on thymocyte development and T cell activation. Our data suggests, that the inhibitory effects of SinΔC on T lymphocyte function are mediated by the Crk/C3G complex and Rap1. Rap1 activation in T lymphocytes correlates with reduced ERK activation and reduced transcriptional activation. In addition, the Src kinase Fyn is also required for some of the inhibitory effects of SinDC (manuscript in preparation). Thus, our results suggest that SinΔC may

be exerting its effect through a defined set of substrates that are similar in different cell types. However, although SinΔC associates with similar substrates in the different cell types we have tested (293HEK cells and T lymphocytes) the functional outcome of these interactions is cell type specific. Thus, in 293 cells SinΔC-mediated Src kinase activation has a positive effect leading to activation of the ERK kinase and transcriptional activation, whereas in T lymphocytes SinΔC-mediated Src kinase activation leads to inhibition of ERK phosphorylation and transcriptional activation. Given these observations we believe that it will be important to examine the effect of SinΔC- and Sin-MTV-mediated Src kinase activation on Rap1 activation, ERK phosphorylation and transcriptional activation in a cell system that is relevant to the mammary epithelium. To this end the effect of activation of the Rap1 pathway on cellular transformation and mammary tumorigenesis is currently being explored in mammary epithelium cell lines (184B5 and/orMCF10A) and in the future in c-Src/SinΔC transgenic mice.

In our previous studies we also identified three Sin tyrosine motifs (Y motifs) that are important for Src signaling by recruiting the Crk/C5G/Rap1 signaling complex (3). These Y-motifs contain the conserved amino acid sequence YDVP, become phosphorylated by Src and are important for Src-dependent transcriptional activation. Although our data show that the Y-motifs of Sin, described above, are required for Src signaling in the form of transcriptional activation, at the present time it is not clear whether these motifs and subsequent Rap1 activation are required for Src dependent, Sin-mediated transformation. We are currently testing this possibility in cell cultures using a SinΔC mutant with point mutations on the three YDVP-containing motifs that substitute the tyrosine residues with phenylalanine (SinΔC-TM). If these motifs are important for Sin-mediated Src transformation we should observe no transformation of 184B5 and/orMCF10A cells expressing c-Src and SinΔC. The effect of this mutant will then be expressed in the mammary epithelium of transgenic mice expressing c-Src and SinΔC-TM. If these Y-motifs of Sin are required for Sin-mediated Src transformation we should observe no tumor formation in these mice. If it turns out the Sin-mediated activation of the Rap1 pathway is required for tumorigenesis, Rap1 will then serve as a good target for therapeutic approaches and drug design. If cellular transformation and tumors are still observed, we will perform additional mutagenesis on Sin. These sequences will then be used as probes to identify proteins that bind to them as described in the original proposal to identify sequences that abolish transformation.

In our previous experiments we generated constructs expressing a 92 amino acid fragment of Sin with substitutions on specific residues that modify the ability of this peptide to modulate the activity of Src. In our preliminary experiments we found that certain substituted peptides inhibit the activity of oncogenic Src as assayed by transcriptional activation, while they have no effect on wild type Src. These peptides provide us with the opportunity to develop specific inhibitors for activated Src kinases and perform structure-function analysis to gain insight in how the activity of Src is regulated. However, although these peptides can block oncogenic Src-mediated transcriptional activation, we found that they had no effect on oncogenic Src transformation. When these peptides were coexpressed with either wild type or oncogenic Src they neither induced nor inhibited Src's oncogenic potential.

In similar experiments we tested the potential of shortened, synthetic Sin-derived peptides for their ability to inhibit Src transformation. The reason these shortened

peptides were generated was that the original peptides were too long to be of experimental value. To this end, we have generated shorter peptides that allow easier experimental manipulation based on the design shown in Fig. 3. These peptides are now 21-25 amino acids long and are based on Sin sequences that effect the enzymatic activity of Src

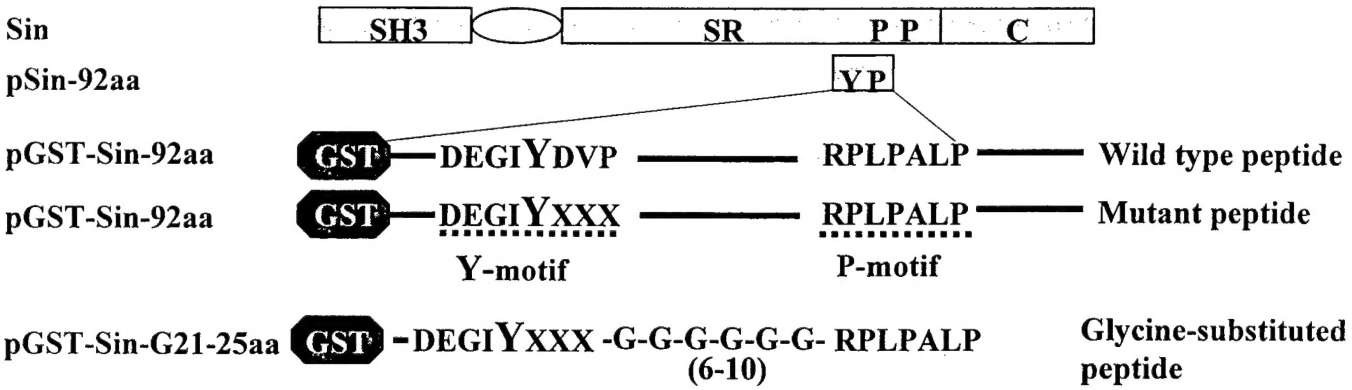

Fig. 2. *Schematic representation of Src inhibitory peptides.* PGST-Sin-92aa is a 92 amino acid fragment of Sin containing tyrosine and proline motifs at the N- and c-termini respectively. The glycine-substituted peptides contain the Y- and P-motifs and 6-10 intervening glycine residues expressed as GST-fusion proteins. X represents amino acid substitutions that modulate the biologic properties (inhibitory or activating for Src) of the peptides.

These sequences consist of one tyrosine-containing (Y-motif) and one proline-rich motif (P-motif). These motifs are found on the N- and C-termini respectively and the intervening sequences (found on Sin) are substituted with 6-10 glycine amino acid residues. The use of glycine residues allows for the generation of shorter peptides and also provides structural flexibility that should allow the tyrosine and proline motifs to bind to the Src SH2 and SH3 domains and modulate Src's activity. However, when we tested these peptides we found that they had no effect on the signaling or oncogenic activity of Src. This was probably due to the inability of these peptides to associate with Src since we could not detect the peptide in Src immune complexes. The inability of the peptides to associate with Src could in turn be due to structural constraints that prevent association of the proline-rich and tyrosine containing motifs of the peptide with the conserved SH2 ans SH3 domains of Src. Given these results we have now abandoned this project. Instead, we will concentrate our efforts into identifying protein substrates that interact with Sin and prove their involvement in Src-mediated tumorigenesis.

KEY RESEARCH ACCOMPLISHMENTS

1. We have shown that Sin interaction with Src leads to the activation of Src signaling as assayed by transcriptional activation.
2. We have for the first time described a novel pathway that mediates wild type c-Src signaling.
3. We have confirmed that this pathway operates in vivo downstream of Src kinases using thymocytes and T cells as a model system

4. We have characterized the components of this pathway both in vitro and in vivo. These include Src/Sin, the signaling complex Crk/C3G, the G-protein Rap1 and the kinase ERK1.
5. Our experiments have revealed mechanistic differences in the signaling mechanisms of wild type versus transforming Src alleles.

REPORTABLE OUTCOME

-We have published a manuscript under the title "c-Src Signaling induced by the adapters Sin and Cas in mediated by Rap1 GTPase". 2000. Mol. Cell. Biol. Vol. 20: p. 7363-7377.
Another manuscript is currently under preparation describing the function of SinΔC in T lymphocyte development and function.

An oral presentation and an abstract were presented in an annual meeting: "Tyrosine Phosphorylation and Cell Signaling: The Third Decade". August 9-13, 2000, The Salk Institute, San Diego, CA.

An oral presentation was given at the FASEB annual meeting: "FASEB 2000, Signal Transduction in the Immune System". Saxton River, VT.

An oral presentation and two abstracts were presented at "The Fortieth Midwinter Conference of Immunologists: Immune System Development and Function" , January 27-30, 2001, Asilomar, CA

An oral presentation was presented at the International Symposium on: "The Molecular Basis of Immune Cell Activation and Immunological Disorders", February 15-18, 2001, San Diego, CA

-No degrees obtained

-We have developed cell lines coexpressing Src and SinΔC using NIH3T3 cells.

-No informatics

-We have received funding from the National Institute of Allergy and Infectious Diseases (NIAID) on work supported by this award

RO1 AI49387 $1,125,000
Title: Examine the Role of Fyn and Rap1 in T cell activation and T cell-mediated Immune Responses.

-No changes in employment status.

CONCLUSIONS

Oncogenic Src proteins have been extensively studied to gain insight into the signaling mechanisms of Src. To better understand signaling through wild-type Src, we used an approach that involves activation of Src signaling through the binding of physiologic ligands to the Src SH3 domain. To this end we used full length and truncated versions of the multi-adapter Sin to activate c-Src, and we examined the intracellular pathways that mediate Src signaling under these conditions. We found that Sin-induced Src signaling, as assayed by transcriptional activation, is exclusively mediated through a pathway that involves the adapter Crk and the GTP-binding protein Rap1. Activation of the Rap1 pathway by Sin was mediated by three conserved sequences within Sin that promote association of Src-phosphorylated Sin with the Crk/C3G/Rap1 signaling complex. The involvement of this pathway downstream of Src kinase-phosphorylated SinDC was confirmed in vivo using thymocytes and T cells as model systems. These data are in contrast to previous observation showing that another GTP-binding protein, Ras, mediates signaling downstream of transforming Src alleles. In our system we found that signaling through an oncogenic Src allele is indeed mediated by Ras. In addition, we found that Rap1 also mediates oncogenic Src signaling.

These results are important because for the first time implicate the Rap1 pathway downstream of activated wild-type Src and provide insight into the signaling mechanisms of this protein. Our results are also important because for the first time they reveal mechanistic differences in the signaling properties of wild-type and transforming Src alleles. In addition, our results reveal Sin as an important player in Src kinase signaling. Insight provided by these experiments into the molecular mechanisms of Src signaling will be of great value in addressing the signaling events that lead to Src dependent transformation and tumorigenesis in the mammary epithelium.

REFERENCES

1. **Alexandropoulos, K., and D. Baltimore.** 1996. Coordinate activation of c-Src by SH3- and SH2-binding sites on a novel p130Cas-related protein, Sin. Genes Dev **10:**1341-55.
2. **Brown, M. T., and J. A. Cooper.** 1996. Regulation, substrates and functions of src. Biochim Biophys Acta **1287:**121-49.
3. **Xing, L., C. Ge, R. Zeltser, G. Maskevitch, B. J. Mayer, and K. Alexandropoulos.** 2000. c-Src signaling induced by the adapters sin and cas is mediated by rap1 GTPase [In Process Citation]. Mol Cell Biol **20:**7363-77.

APPENDIX